Journal of Medical Virology 55:268–271 (1998)

Human Cytomegalovirus Infection Induces mRNA Expression and Secretion of Plasminogen Inhibitor Type-1 in Endothelial Cells

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The aim of the study was to investigate whether infection of endothelial cells with human cytomegalovirus (HCMV) perturbs expression and production of plasminogen activator inhibitor type-1 (PAI-1). mRNA expression of PAI-1 was investigated by isolating total RNA from HCMV-infected and control cells, followed by Northern blotting and probing with ^{32}P-labelled PAI-1 probe. Sandwich ELISA was used to investigate PAI-1 production. HCMV induced the expression of PAI-1-mRNA at 2–5 days postinfection (maximum expression was at 3 days postinfection which was 40% higher than control). HCMV also induced secretion of PAI-1 at 2–5 days postinfection. These results indicate that infection of endothelial cells with HCMV disturbs PAI-1 expression and production in these cells. ***J. Med. Virol. 55:268–271, 1998.***

KEY WORDS: angiogenesis; vascular cells; proteolytic enzymes

INTRODUCTION

It is well-documented that a main feature of healthy blood vessels is that their endothelial cells are in a nondividing condition [Ross, 1993]. Previous work has shown that infection of endothelial cells with a clinical isolate (C3) of human cytomegalovirus (HCMV) induced proliferation of these cells and enabled them to maintain growth in media deficient in growth factors, a necessary requirement for normal endothelial cell growth in vitro [Woodroffe et al., 1993]. Hence, HCMV induction of endothelial cell proliferation is very important since it contributes to angiogenesis-dependent diseases such as atherosclerosis, hypertrophy infarction, solid tumours invasion (metastasis), arthritis, Kaposi's sarcoma [Boldogh et al., 1990; Brem et al., 1993]. Investigation of the mechanisms involved in HCMV-induced endothelial cell proliferation therefore will enable us to understand the biology of HCMV infection and its possible role in many angiogenesis-dependent diseases.

Factors contributing to HCMV-induced endothelial cell proliferation include production of inhibitors for proteolytic enzymes such as the plasminogen activator enzyme system, which is able to influence activation of basic fibroblast growth factor (bFGF) and transforming growth factor β (TGFβ) [Saksela and Rifkin, 1990; Peacock et al., 1995]. It has been reported that infection of human and hamster cells with HCMV induced production of plasminogen activator enzyme [Yamanishi and Rapp, 1979], however, production of inhibitors for these enzymes after infection, has not been investigated. The inhibitor, plasminogen activator inhibitor type-1 (PAI-1) produced mainly by vascular endothelial cells and smooth muscle cells, inhibits the action of two predominant plasminogen activator enzymes, u-PA and t-PA, which catalyse the cleavage of plasminogen to plasmin [Vassalli et al., 1991]. Hence, PAI-1 is considered one of the major factors affecting cell proliferation by regulating the plasminogen activator enzymes, indirectly affecting bFGF level [Vassalli et al., 1991] and by inhibiting the activation of TGFβ, a potent suppressor for vascular cell proliferation [Grainger et al., 1994]. Recent reports have also suggested that increased production of PAI-1 promotes angiogenesis by inducing endothelial cell migration [Grainger et al., 1994].

To our knowledge, there has been no investigation of the effect of infection of endothelial cells with a clinical isolate of HCMV on mRNA regulation and production of PAI-1. This study describes mRNA expression and production of PAI-1 by human endothelial cells after infection with HCMV.

Contract grant sponsor: the National Health and Medical Research Council of Australia.

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Accepted 11 February 1998

MATERIALS AND METHODS

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cord vein [Jaffe et al., 1973]. Cultures were propagated in endothelial cell growth medium, consisting of M199 (ICN, Sydney, Australia) supplemented with 20% heat inactivated foetal calf serum (FCS, CSL, Sydney, Australia), 100 μg/ml endothelial cell growth supplement (ECGF), 90 μg/ml heparin (Sigma, St. Louis, MO), 110 μg/ml sodium bicarbonate, 2 mM L-glutamine, 20 mM HEPES, 10 μg/ml penicillin, and streptomycin (Cytosystems, Sydney, Australia).

Virus

HCMV strain AD169 was initially purchased from the American Type Culture Collection VR-533.

A clinical isolate (C3) which was described fully in a previous report [Woodroffe et al., 1993], was isolated from the urine of a 1-year-old infant with histologically-verified HCMV infection and was propagated in endothelial cells. It has ability to induce HUVEC proliferation and react positively with a cocktail of two monoclonal antibodies against HCMV 43 and 76 kDa proteins (DAKO, Australia). These monoclonal antibodies also reacted positively with fibroblasts infected with the laboratory strains AD169 of HCMV (data not shown). C3 was tested for mycoplasma and all primary cultures were routinely treated with BM cyclin and checked for mycoplasma using DAPI stain (Boehringer Mannheim, Sydney Australia).

UV-Inactivation of Virus Stock

C3 stock was placed in a small petridish (35 mm diameter) exposed to 80,000 ergs/mm^2 of mid-range (250–400 nm) UV-light on ice for 20 min and was used to infect HUVEC in a 25 cm^2 flask and a 24 well plate. The absence of CPE, (15 days postinfection) and HCMV nuclear antigen (24 hr postinfection) was investigated to ensure that the UV-inactivation was successful. UV-treated mock was prepared in the same manner.

Northern Blotting

For mRNA analysis, HUVEC were used at early passage numbers (3–5 passages) when they exhibit optimal cell division. HUVEC were seeded at 10^5 cells in 75 cm^2 flasks (Nunc, Inc., Naperville, IL), then were infected with AD169 or C3 at a multiplicity of 1:5 tissue culture infectivity dose 50 ($TCID_{50}$)/cell or 2 $TCID_{50}$/cell or 5 $TCID_{50}$/cell [Woodroffe et al., 1993]. Centrifugation for 20 min at 150*g* and incubation for 2 hr were followed by replacing the inocula with a fresh medium. Mock-infected stock was prepared similar to the C3-infected stock except in the former case, uninfected endothelial cells (HUVEC) were used. Mock- and HCMV-infected cells were lysed using total RNA isolating reagent (Integrated Science, Australia) followed by phenol-chloroform extraction. Northern analysis was performed on 20 μg total RNA prepared from AD169 or C3- or mock-infected HUVEC separated on 1.2% agarose gel (Sigma) containing 2.5% formaldehyde and capillary-transferred to nylon membrane (Amersham, Arlington Heights, IL). Hybridisation was performed using ^{32}P-labelled cDNA fragments (random primer method) of either the housekeeping gene probe (18s rRNA) or human PAI-1 cDNA probe kindly supplied by Dr. Loskutoff (Scripps Clinic and Research Foundation, La Jolla, CA). The intensity of mRNA bands for PAI-1 in the mock and C3- infected HUVEC was analysed using an image densitometer analysis program (Biorad, Sydney, Australia).

Enzyme-Linked Immunosorbent Assay (ELISA)

To investigate whether HCMV infection of HUVEC produces post transcriptional changes of PAI-1, a specific and sensitive sandwich ELISA (American Diagnostic, Greenwich, CT), was used to detect PAI-1 levels in the culture media and cell lysate of mock- and C3-infected HUVEC (after 1, 2, 3, 4, 5, 8 days postinfection). In these experiments HUVEC were seeded (in triplicate) at 1×10^4 cells/well in 24 well plates followed by infection with C3 or mock-control at moi 5:1 ($TCID_{50}$/cell). At the indicated times post infection the supernatant as well as three washes of the monolayer of each well were collected and stored at −80°C. The monolayers were lysed using 1% SDS solution and 2 hr agitation on ice then were stored at −80°C.

RESULTS

Infection of endothelial cells with C3 at a multiplicity of 1:5 $TCID_{50}$/cell produced clear nuclear antigen in very few cells (Fig. 1). However, infection of endothelial cells with C3 at a multiplicity of 5:1 $TCID_{50}$/cell produced nuclear antigen in nearly 80% of the cells and induced clear changes in the expression of mRNA for PAI-1. The Northern blot described in Figure 2a shows the distinctive two bands of PAI-1 at 3.2 and 2.2 Kb corresponding to the previously described human PAI-1 in HUVEC [Christ et al., 1993]. As shown in Figure 2a, C3 induced expression of mRNA for PAI-1 at 3 days (lane 2) and 5 days (lane 3) postinfection when compared with mRNA expression in mock-infected HUVEC at 3 days (lane 5) and 5 days (lane 6) post infection. Figure 2b represents the densitometric data of PAI-1 3.2 kb mRNA (Fig. 2a) corrected to the intensity of the corresponding 18s hybridisation intensity signal. The Y axis of Figure 2b represents percentage intensity of PAI-1 bands -of C3- and mock-infected cells- divided by the corresponding intensity signal of the 18s bands. Similar results were obtained when the 2.2 kb mRNA for PAI-1 was used in comparison to 18s species (data not shown). Figure 2b confirms that C3 induced maximum expression of PAI-1 at 3 days postinfection. In another set of experiments it was noticed that C3 induced low level of mRNA expression for PAI-1 at 2 and 4 days postinfection (data not shown).

Results shown in Figure 3 suggest that C3 induces an increase in the level of released PAI-1 after 2–5 days postinfection ($P = 0.005$ for day 3 postinfection), which returns to the control level at 8 days postinfection.

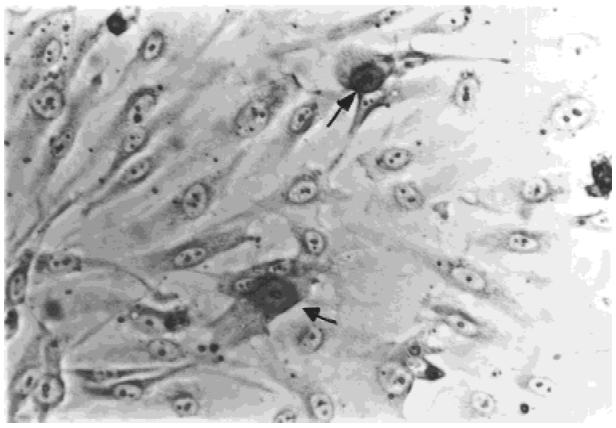

Fig. 1. Nuclear antigen expression of the clinical isolate of HCMV (C3) in HUVEC at 24 hr postinfection (arrows) as demonstrated by immunophosphatase staining. HUVEC were infected with C3 at multiplicity of 1:5 $TCID_{50}$/cell.

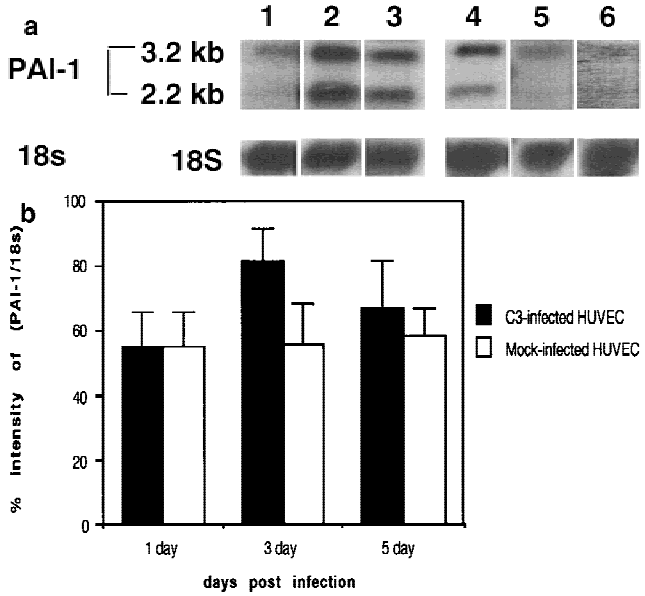

Fig. 2. **a:** Densitometer image for the expression of mRNA for PAI-1 in mock and HCMV-infected HUVEC. Endothelial cells were infected with mock or C3 at moi 5:1 $TCID_{50}$/cell, total RNA was extracted from the cells at 1, 3, 5, days postinfection (p.i.). Lane 1 represents C3-infected cells at 1 day p.i, lane 2 represents C3-infected cells at 3 days p.i., lane 3 represents C3-infected cells at 5 days p.i, lane 4 represents mock-infected cells at 1 day p.i, lane 5 represents mock-infected cells at 3 days p.i., and lane 6 represents mock-infected cells at 5 days p.i. Experiment was repeated twice. **b:** mRNA expression of PAI-1 as % of the intensity of the corresponding 18s hybridisation signal. C3-infected cells (■), Mock-infected cells (□).

Meanwhile, no difference in PAI-1 level could be detected in the cell lysate of mock- or C3-infected cells (data not shown).

DISCUSSION

In this study, a clinical isolate (C3) of HCMV were used to infect human umbilical vein endothelial cells (HUVEC). C3 was able to infect endothelial cells efficiently, producing clear cytopathic changes and high

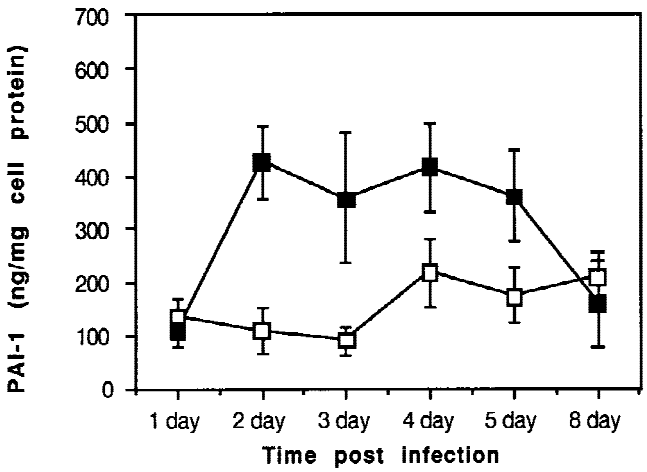

Fig. 3. The production of PAI-1 in the supernatant of mock- and HCMV-infected HUVEC. HUVEC were infected with mock or C3 at moi 5 TCID50/cell and PAI-1 secretion was detected using sandwich ELISA kit. C3-infected cells (■), Mock-infected cells (□). The readings represent the mean of two experiments with each test done in triplicate. Bars represent the standard error.

level of HCMV nuclear antigens (Fig. 1). However, HUVEC infected with AD169 did not show any cytopathic changes or any nuclear antigen (data not shown). This confirms previous reports suggesting that the laboratory strains of HCMV such as AD169 are unable to efficiently infect endothelial cells even when high multiplicities of infection were used (only 2–10% of cells express HCMV nuclear antigen and no cytopathic changes on these cells are produced), [Alcami et al., 1991; Waldman et al., 1991; Woodroffe et al., 1993]. Additionally, it has been shown that these clinical isolates are able to induce changes in the normal functions of endothelial cells which are different or at a different level to that induced on endothelial cells by the laboratory strain AD169 of HCMV. For example, it has been shown that infection of HUVEC with clinical isolates increased their transformation [Smiley et al., 1988], and proliferation [Woodroffe et al., 1993], while the laboratory strain, AD169, failed to do so [Alcami et al., 1991]. Also the clinical isolate (C3), rather than AD169, affects HUVEC properties such as cytokine production and the expression of integrin receptors for fibronectin, collagen, and laminin [Shahgasempour et al., 1997].

HUVEC were used at early passage numbers (3–5 passages) when they exhibit optimal cell division. Since it is not feasible to obtain enough actively dividing HUVEC to be able to investigate mRNA expression after each day postinfection, it was decided to restrict the investigation to alternate days postinfection. The above results suggest that HCMV infection of HUVEC induces an increase in the level of mRNA expression for PAI-1 at 2–5 days postinfection with optimal production at 3 days postinfection. These induced changes may be due in part to the HCMV induction of factors affecting mRNA stability. However, treatment of HUVEC with C3-free supernatant did not induce increase in the expression of mRNA for PAI-1 in these cells and

the level of PAI-1 expression was similar to that of mock-infected HUVEC (data not shown). This suggests that these changes in mRNA are not due to soluble factors produced by the virus in the supernatant. Similarly, infecting HUVEC with the laboratory strain AD169 or treatment of HUVEC with UV-inactivated C3 did not induce changes in mRNA expression (data not shown), suggesting that active infection or replication of HCMV are needed for the induced changes in PAI-1 mRNA expression. This may be due to the expression of HCMV immediate early/early regulatory genes which may affect PAI-1 gene promoter or elements in its promoters. This could be confirmed by a different set of experiments which is beyond the scope of the present study.

The data presented above also indicate that infection of HUVEC with HCMV clinical isolate (C3) induces increase in the secreted levels of PAI-1 at the early stages of infection (2–5 days postinfection). This was followed by a decrease in secretion of PAI-1 at late stages of infection (7–8 days). Similarly, C3-free supernatant and UV-inactivated C3 or infection with AD169 did not induce any changes in PAI-1 production, further suggesting that HCMV-gene expression is needed for PAI-1 regulation and secretion (data not shown). It has been reported that infection of endothelial cells with heat-inactivated AD169 did not change PAI-1 activity. However, there was a very slight decrease in PA-1 activity at 50 hr postinfection [van Dam-Mieras et al., 1994]. These results support our suggestion that active virus infection is needed to induce changes in PAI-1 secretion.

HCMV-induced changes in PAI-1 level in endothelial cells may affect other factors such as the production of active TGFβ, which is a potent inhibitor of endothelial cell proliferation [Grainger et al., 1994], and the proteolytic enzymes t-PA and u-PA [Vassalli et al., 1991]. Recently it has been reported that infection of fibroblasts with HCMV laboratory strain AD169 induced the transcription and secretion of TGFβ at 24 hr postinfection [Michelson et al., 1994] and caused a slight increase in secreted plasminogen proteolytic enzymes u-PA after 24 hr infection with AD169 [van Dam-Mieras et al., 1994].

In conclusion, we show that infection of endothelial cells with HCMV affects the mRNA expression and secretion of PAI-1 which are due to direct virus replication. PAI-1 is considered a key-regulatory factor involved in endothelial cell growth, proteolysis, and angiogenesis. Hence, perturbation of this enzyme after infection of endothelial cells with HCMV may contribute to many disease conditions in particular angiogenesis-dependent diseases.

ACKNOWLEDGMENTS

We thank Dr. Loskutoff for the gift of cDNA probe for PAI-1 and Dr. Marie Ranson for the housekeeping gene and for technical assistance.

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